Analytical Biochemistry 696 (2025) 115692

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

The downstream purification of bispecific antibodies

Qian Li [1], Hongyang Zhao [1], Xiaoying Liang, Qingquan He, Zicheng Wang, Guohong Qin, GuoZhu Li, Dan Xu [*]

Nanjing Chia-Tai Tianqing Pharmaceutical Co.Ltd, Fanghua Research Institute, Institute of Biology, Nanjing, 210046, China

ARTICLE INFO

Keywords:
Bispecific antibody (bsAb)
Capture chromatography
Product-related impurities
Process-related impurities
Removal strategies

ABSTRACT

Bispecific antibodies, a class of therapeutic antibodies, can simultaneously bind to two distinct targets. Compared with monospecific antibodies, bispecific antibodies offer advantages, including superior efficacy and reduced side effects. However, because of their structural complexity, the purification of bispecific antibodies is highly challenging. The purification process must strike a delicate balance between purity and productivity, eliminating a broad spectrum of contaminants, including product-related and process-related impurities, while also maximizing the yield wherever feasible. This review systematically describes the strategies for bispecific antibody capture, the elimination of product-related impurities, and the mitigation of the formation of process-related impurities, thereby, providing guidance for the development of downstream purification processes for bispecific antibodies.

1. Introduction

Bispecific antibodies (bsAbs) are artificially synthesized molecules that can simultaneously bind to two distinct targets [1,2]. Compared with monoclonal antibodies, bsAbs offer several advantages, including increased specificity, potentially reduced side effects, and the ability to engage multiple pathways or cells in a targeted manner for enhanced therapeutic outcomes [3]. To date, there are 14 bsAbs that have been approved for marketing, with over a hundred different types of bsAbs in various preclinical development stages [4–6]. Thus, the development of bsAb is a current topic of interest in the research and development of biopharmaceuticals.

As shown in Fig. 1, bispecific antibody structural designs have become increasingly diverse. bsAbs can be systematically grouped into two categories according to the structures: IgG-like bsAbs and non-IgG-like bsAbs [7]. IgG-like bsAbs generally exhibit good stability and a longer half-life in vivo, although their larger size can limit the tissue penetration capacity [7]. Conversely, non-IgG-like bsAbs, because of their smaller molecular structure, often display enhanced tissue penetration but can potentially have shorter half-lives [8]. Additionally, bsAbs can be categorized into symmetric bsAbs and asymmetric bsAbs [7,8]. The different bsAbs structures of bsAbs necessitate tailored purification strategies to meet both quality and yield requirements.

As shown in Fig. 2, the downstream purification steps for bsAbs include capture, low pH viral inactivation, intermediate purification, polishing, virus clearance filtration, and ultrafiltration diafiltration [9]. Each of these steps is designed to target and eliminate specific classes of contaminants [9]. The purification processes needs to remove two different types of impurities: product-related impurities and process-related impurities [1]. Product-related impurities include homodimers, fragment contaminants, and aggregates, and process-related impurities contain cell host proteins, viruses, endotoxins, and other such substances [1]. Li et al. and Chen et al. published comprehensive reviews on the downstream purification of bsAbs, in 2019 and 2021 respectively [1,8,10]. However, there are not many comprehensive reviews that systematically describe the overall solution strategies for downstream purification of bispecific antibodies. In this review, we summarize the chromatographic methods employed for the capture of bsAbs and provide a perspective on alternative affinity chromatography approaches. Furthermore, we systematically outline the strategies used to remove both product-related and process-related impurities in detail. This review provides a theoretical guide for the development of downstream purification processes for bispecific antibodies.

2. Capture chromatography of bispecific antibodies

Capture chromatography is a crucial step of the downstream

* Corresponding author.
E-mail address: liqian12536@dingtalk.com (Q. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ab.2024.115692
Received 5 September 2024; Received in revised form 17 October 2024; Accepted 17 October 2024
Available online 18 October 2024

purification processes for bsAbs, especially given the considerable increase in the expression of bsAbs obtained from Chinese hamster ovary cell (CHO) over the last decade [11]. Currently, affinity chromatography (AC) is the favored capture method, primarily because of the remarkable specificity [12]. As depicted in Fig. 3, two distinct types of affinity chromatography resins are used for capturing antibodies. The first type is affinity ligands that target the heavy chain (HC) region of antibodies, which have proved efficacious in capturing IgG-like bsAbs [10]. Protein A affinity chromatography, a HC-binding affinity chroamtography, has been extensively adopted for capturing antibodies [11]. Tang et al. have used MabSelect SuRe LX, a protein A resin produced by Cytiva, to effectively capture asymmetric IgG-like bsAb with yield of 94.2 % at 30 g/L loading capacity [13]. To cater for the increasingly high titers of bsAbs produced using CHO cell line, protein A resin with high binding capacities such as MabSelect prism A have been developed [14]. MabSelect prism A not only binds to the HC region but also exhibits affinity for the VH3 domain [10]. Pabst et al. have reported that the dynamic binding capacity of MabSelect prism A can reach 58–74 mg/ml at 2–4 min residence time [9]. Protein G affinity chromatography is another affinity resin that possesses HC-binding activity [1]. However, protein G affinity chromatography possesses a low binding capacity and is less stable than protein A affinity chromatography during the elution steps [15]. Consequently, few studies have used protein G affinity chromatography to capture bsAbs. Moreover, to address the drawback with most HC-binding affinity chromatography resins that require a low pH for target antibody elution [1]. HC-binding affinity ligands, CaptureSelect CH1-LX, have been developed that can bind to the CH1 of IgG [10]. A study has demonstrated that using CaptureSelect CH1-LX allowed bsAbs to eluated at pH 4.0 instead of 3.6, which is the pH required for using protein A resin [16].

The second type of affinity chromatography resins are required because no-IgG like bsAbs lack the Fc region. Consequently, using affinity ligands that can interact with the light chain (kappa or lambda chain light chain) is necessary [10]. As illustrated in Fig. 3, protein L affinity chromatography ligands can bind to the variable region of the kappa light chains (LCs) [17]. Additionally, the Kappaselect and LambdaFabselect ligands show affinity for kappa LC and lambda LC, respectively [8]. Compared with HC-binding affinity ligands, LC-binding affinity ligands generally exhibit lower binding capacity and require hasher conditions for elution [8]. Therefore, LC-binding affinity ligands

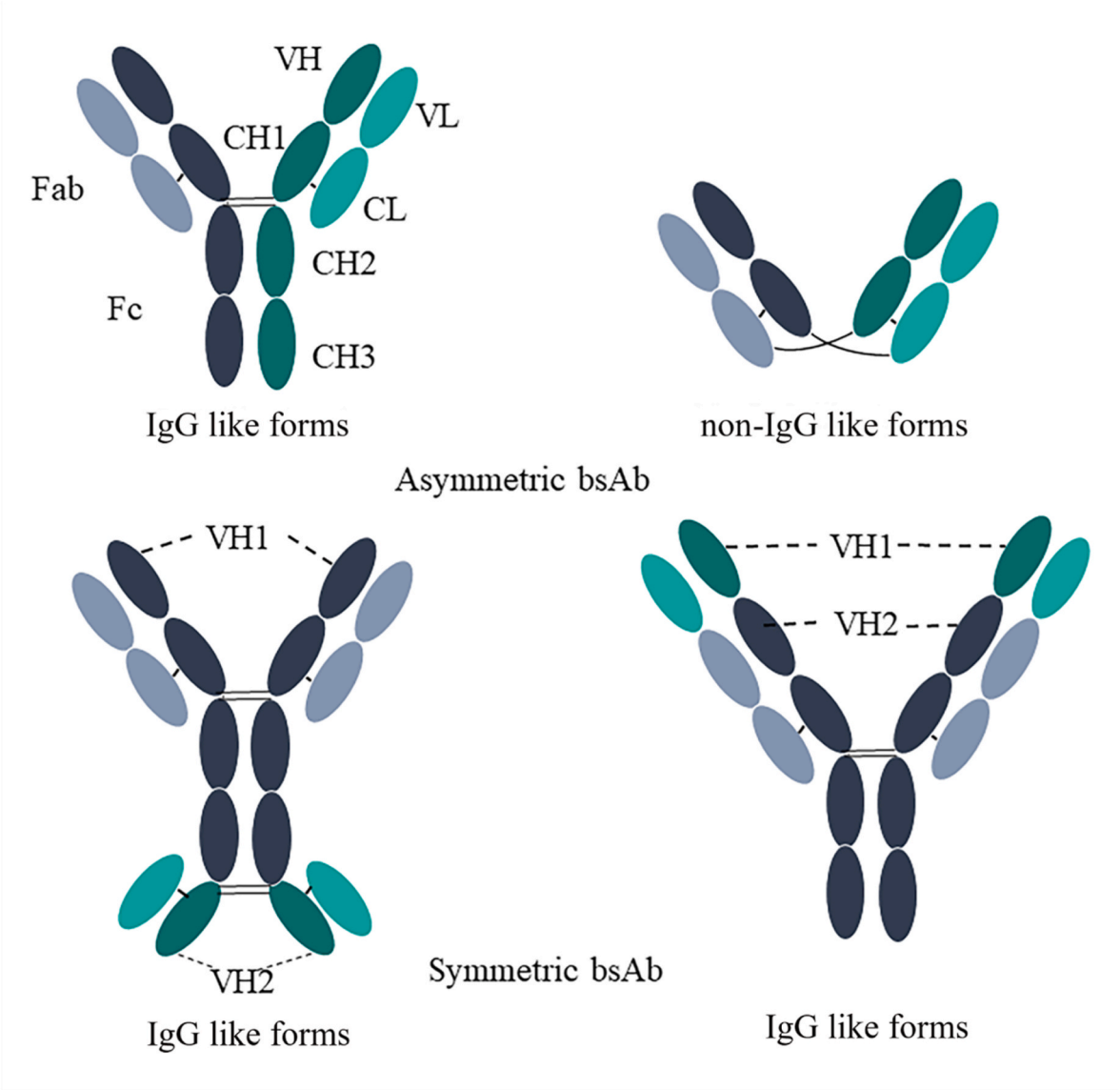

Fig. 1. Schematic representation of bsAb. In terms of structural symmetry, bispecific antibodies can be classified into symmetric and asymmetric types. With respect to the integrity of their structure, they can be categorized into IgG-like and non-IgG-like forms.

are primary used in removing by-products rather than for capturing antibodies directly [8]. The ability of affinity chromatography to remove byproducts will be discussed in detail in subsequent sections.

To sum up, affinity chromatography, especially protein A affinity chromatography, is the gold-standard method for capture bsAb [11]. However, the protein A affinity resin has many inherent drawbacks, including that the price of resin is expensive, the binding capacity is relatively low, and the elution steps require harsh acidic conditions [11]. In recent years, various alternatives to protein A affinity chromatography have been explored [11]. Mix-mode chromatography, which can provide multimodal interactions, can capture bsAbs through non-specific binding [18]. Jerome et al. have evaluated four mix-mode resins for the capture of antibodies and, the results indicated that mix-mode chromatography PPA HyperCel could effectively remove host cell protein (HCPs) (>60 % removal rate) while capturing antibodies with a yield of 93 % [19]. In addition, diverse biomimetic small peptide affinity ligands, have been designed to capture antibodies [20]. Compared with protein A ligands, biomimetic small peptides show higher stability and allows the antibodies to be eluted at milder condition [20]. Barroedo et al. have reported that the peptide Ac-PHQGQHIGVSK could capture antibodies with 98 % purity in 94 % yield [21]. Affinity membrane chromatography has also demonstrated potential for capturing antibodies [22]. Compared with conventional protein A column chromatography, affinity membrane chromatography has a higher throughput and thus a shorter cycle time [22]. Brämer et al. have used Sartobind® Protein A to capture bsAbs [23]. Membrane chromatography ensures high sample purity while reducing the process time by 70 %, compared with conventional protein A column chromatography [23]. Although these technologies have not been widely adopted for industrial-scale bsAbs capture, it is possible that these techniques will revolutionize the process of downstream purification in the future.

3. Removal strategies for product-related impurities

The presence of product-related impurities, such as homodimers, fragments, and aggregation, is an issue for product safety (Fig. 4) [1]. Additionally, because many product-related impurities share similar physicochemical properties with the target bsAbs, the removal of these contaminants can be challenging [8]. This section provides detailed strategies for the removal of product-related impurities (Table 1).

3.1. Removal of homodimers

Bispecific antibodies, which are composed of four distinct polypeptide chains, can recognize and target two different antigens [1]. However, because the expression levels of different polypeptide chains are difficult to control at the same time, mispaired products, especially homodimers, are often produced during the production of bsAb [8]. Although the use of various platform techniques, such as knob into hole (KIH) techniques, can reduce the probability of HC-HC mispairing, homodimers can still constitute up to 5 % of the total mass of product in some cases [7]. Thus, it is crucial to remove homodimers by purification. In this section, we focus on the chromatography technologies for the removal of homodimers.

3.1.1. Affinity chromatography

Affinity chromatography, which uses the difference in binding affinity toward affinity resins between partially bispecific antibodies and homodimers, has been proven to be an effective tool to remove homodimers.

In one method, bsAbs designed that incorporate various modifications aimed at adjusting the interaction affinity between the Fc regions and Protein A [24]. Consequently, a disparity in binding potential to protein A affinity resins arises between the homodimers and the bsAbs

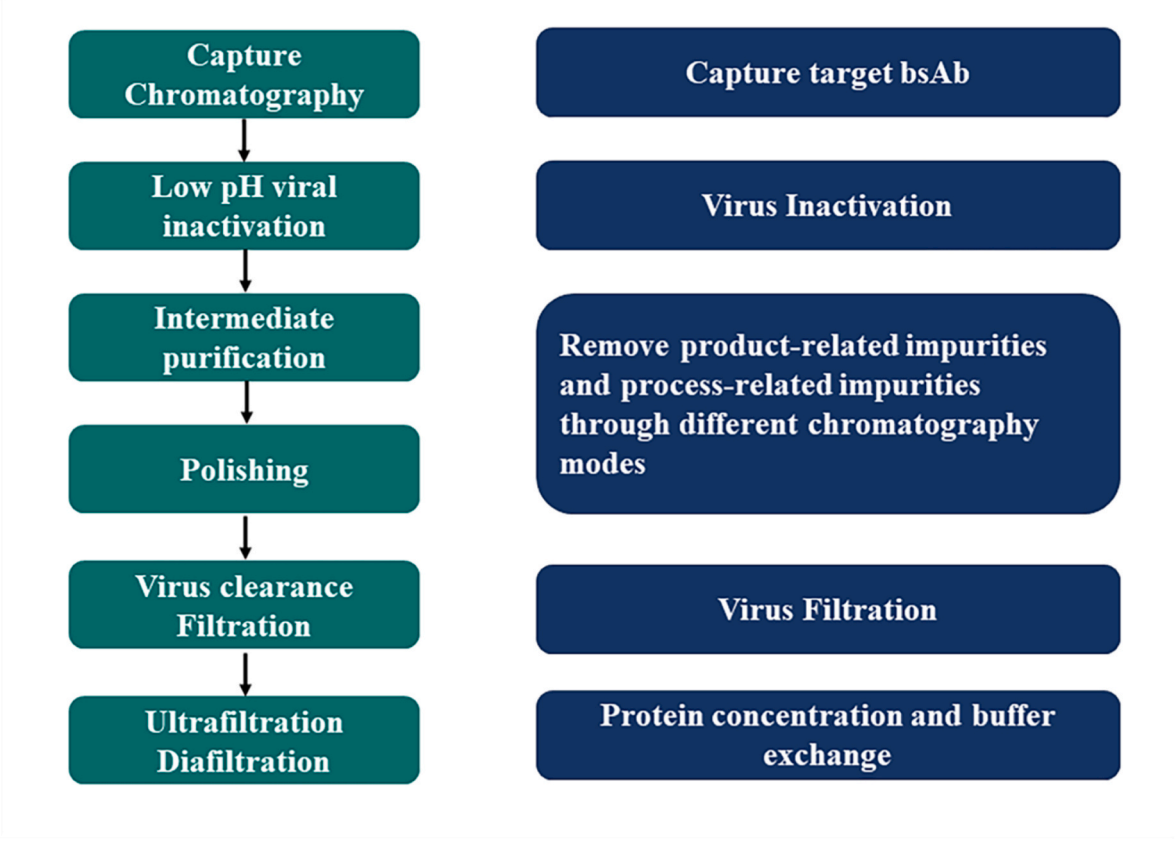

Fig. 2. Schematic diagram of the downstream purification process for bispecific antibodies.

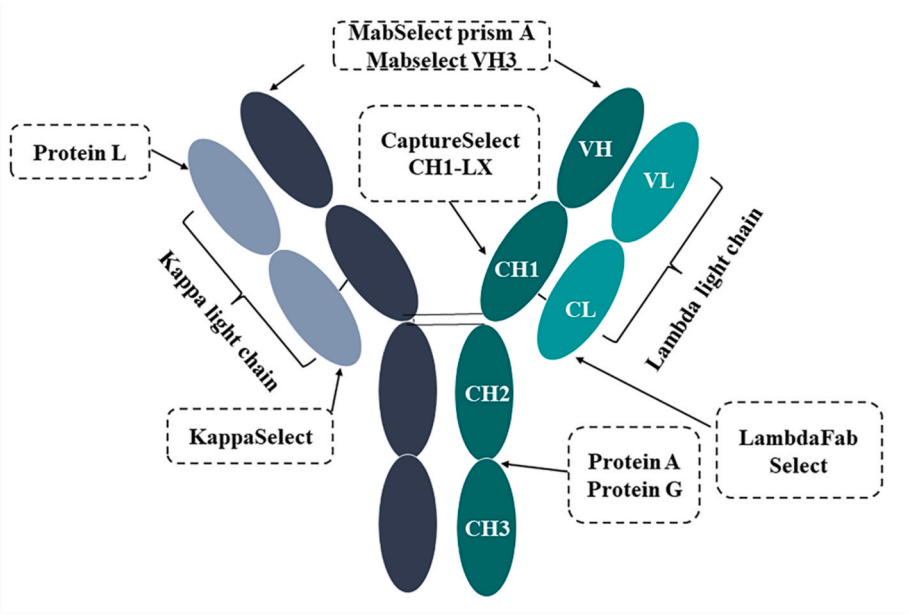

Fig. 3. Binding sites of several affinity ligands. Protein A and protein G resins bind to Fc region. In addition, novel protein A resin MabSelect prism A binds to Fc and VH regions at same time. Mabselect VH3 specifically binds to the VH3 region. CaptureSelect CH1-LX binds to CH1 region. KappaSelect and LambdaFabSelect bind to CL region of Kappa light chain and Lambda light chain, respectively. Protein L binds to VL region of Kappa light chain.

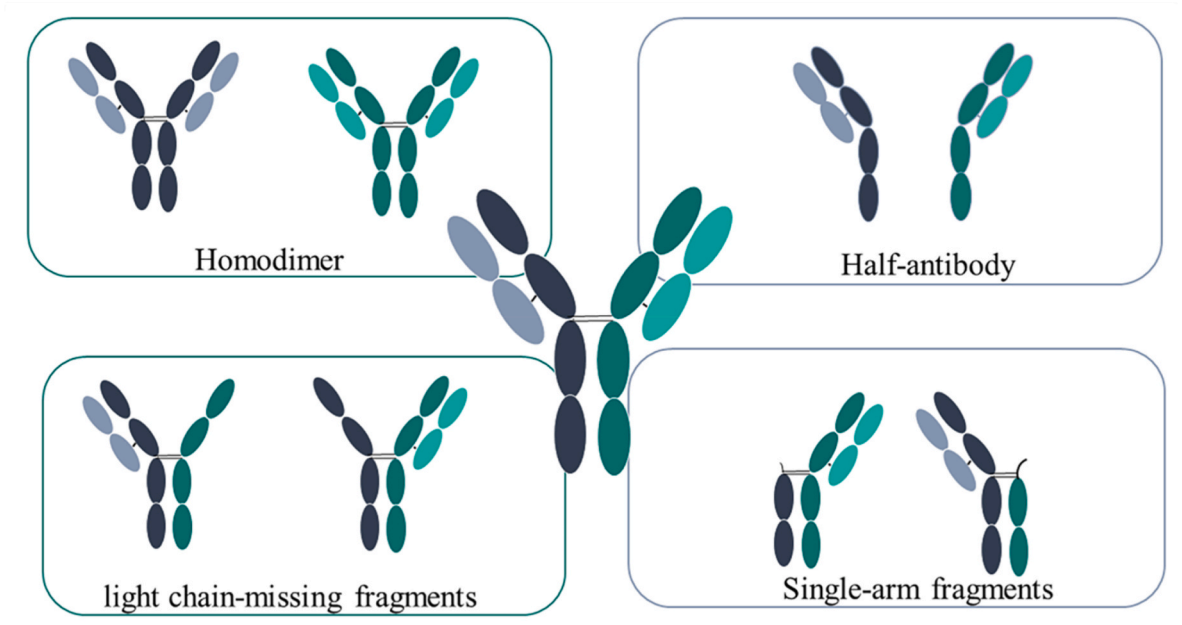

Fig. 4. Schematic diagram of product-related impurities of bsAb.

[8]. For example, in the DuetMab platform, a bsAb platform designed by AstraZeneca, one of the HC (designated Fc*) undergoes alteration to preclude protein A binding [24]. As shown in Fig. 5A, the resultant Fc* Fc* homodimers are unable to bind with protein A and are consequently removed in the flow-through during chromatography [24]. Conversely, FcFc homodimers display a higher affinity for Protein A relative to the bsAb (Fc*Fc) [24]. Therefore, protein A can effectively separate the bsAb from homodimers [24]. Studies have demonstrated that adjusting the salt content in the elution buffers to modulate the hydrophobic interactions can considerably enhance the separation efficiency [24]. Tustian et al. have incorporated $CaCl_2$ into the elution buffer, which markedly improved the resolution between the bsAbs (FcFc*) and the homodimer (FcFc), ultimately achieving a bispecific purity of 95 % [24].

A second approach for affinity chromatography that exploits the differential affinity for the heavy chain variable region (VH) is an efficacious method for eliminating homodimers [10]. Chen et al. have demonstrated the utility of MabSelect Prism A resin, which capitalizes on its specific VH3 binding capacity to facilitate efficient homodimer removal [25]. In this study, the conformational difference in the VH3 region between the bsAb (FabScFv-KiH) and homodimer, increased the affinity difference which lead to effective homodimer removal [25]. This strategy achieved a high purity of 92.2 % while maintaining a yield of 90.6 % [25]. In addition, the affinity resin Mabselect VH3, which specifically binds to the VH3 domain, exhibits an advantage in separating

Table 1
Summary of product-related impurities Removal Strategies for BsAbs.

Type of impurities	Type of chromatography	Type of resin	Format of bsAb	Removal Mechanisms	Reference
Homodimer	Affinity chromatography	Protein A	IgG-like bsAb (FcFc*)	Differential Affinity in the Fc Region	[24]
		MabSelect PrismA	FabScFv-KiH	Differential Affinity in the VH Region	[25]
		MabSelect VH3	IgG-like bsAb (VH2: VH3)	Differential Affinity in the VH Region	[26]
		CaptureSelect CH1-LX	FabScFv-Fc	Differential Affinity in the CH1 Region	[26]
		KappaSelect	Wuxi Body	Differential Affinity in the light chain	[30]
		Capto L	Wuxi Body	Differential Affinity in the light chain	[31]
		MabSelect VL	Wuxi Body	Differential Affinity in the light chain	[32]
	Ion exchange chromatography	MonoS 10/100 GL	ART-Ig	Surface Charge Differences	[34]
		POROS 50HQ	ART-Ig	Surface Charge Differences (weak partitioning mode)	[5]
	Mix-mode chromatography	Capto MMC ImpRes	KiH (knob into hole)	Surface Charge and Hydrophobicity Differences	[13]
		Capto MMC ImpRes	KiH (knob into hole)	Surface Charge and Hydrophobicity Differences	[38]
		Toyopearl MX-Trp 650 M	κλ-Type bsAb	Surface Charge and Hydrophobicity Differences	[39]
Half-antobody	Affinity chromatography	Protein A	bsAb	Differential Affinity in the CH Region	[41]
Light chain missing fragment	Affinity chromatography	Capto L	Wuxi Body	Differential Affinity in the light chain	[43]
		KappaSelect	Wuxi Body	Differential Affinity in the light chain	[30]
	Mix-mode chromatography	Capto Adhere ImpRes	bsAb	Surface Charge and Hydrophobicity Differences	[45]
		Capto MMC ImpRes	bsAb	Surface Charge and Hydrophobicity Differences	[46]
		Capto MMC ImpRes	bsAb	Surface Charge and Hydrophobicity Differences	[47]
Single-arm fragment	Affinity chromatography	MabSelect PrismA	bsAb	Differential Affinity in the Fab Region	[44]
Aggregation	Affinity chromatography	Protein A	bsAb	Affinity and Hydrophobicity Differences	[49]
		Protein L	bsAb	Affinity and Hydrophobicity Differences	[31]
	Mix-mode chromatography	Capto MMC ImpRes	bsAb	Surface Charge and Hydrophobicity Differences	[51]
		Capto MMC ImpRes	bsAb	Surface Charge and Hydrophobicity Differences	[47]
		Diamond MMC Mustang	bsAb	Surface Charge and Hydrophobicity Differences	[52]

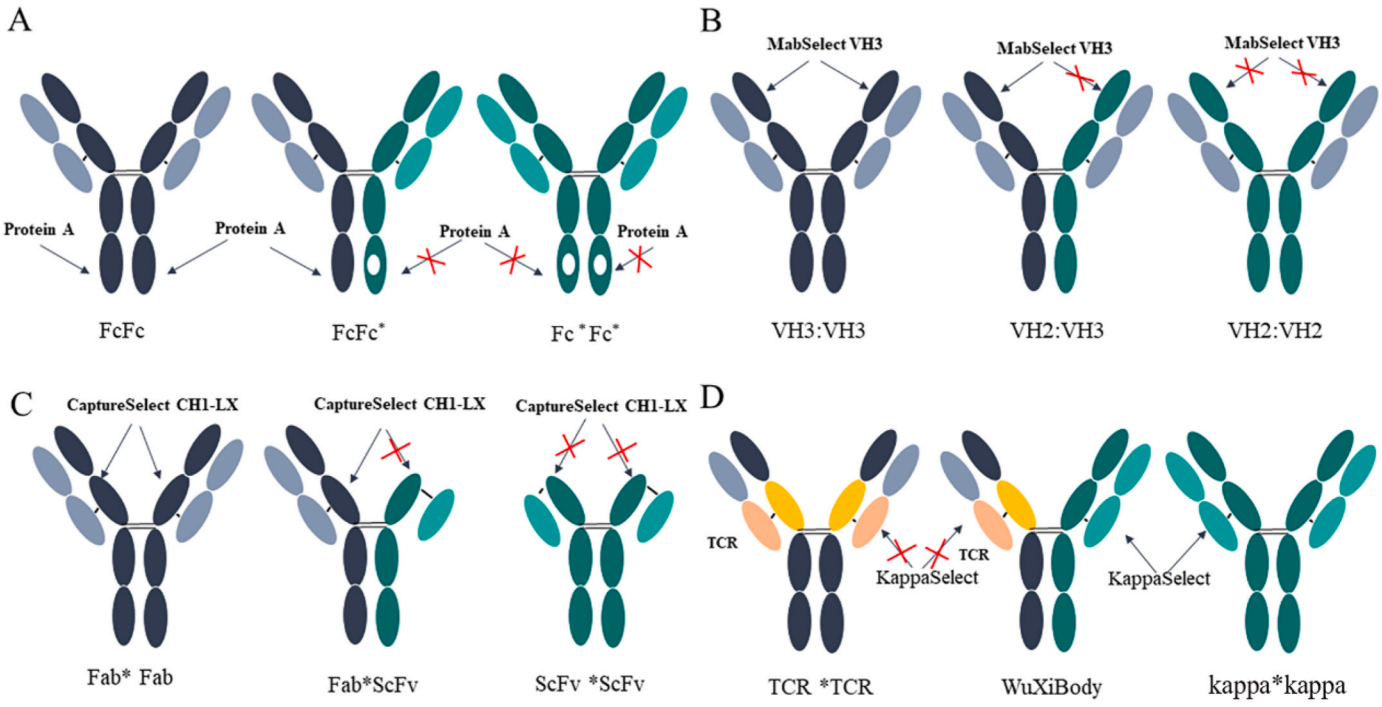

Fig. 5. Schematic strategy for the removal of homodimers using affinity chromatography. **A.** bsAb adopted FcFc* form. Hence, Fc*Fc* cannot bind to protein A, while FcFc bind tightly to protein A. **B.** MabSelect VH3 specifically binds to the VH3 region. Hence, homodimer VH2:VH2 cannot bind to MabSelect VH3, while homodimer VH3:VH3 bind tightly to MabSelect VH3. **C.** CaptureSelect CH1-LX specifically binds to the CH1 region. Hence, homodimer adopted ScFv*ScFv format cannot bind to CaptureSelect CH1-LX, while homodimer adopted Fab*Fab format bind tightly to CaptureSelect CH1-LX. **D.** KappaSelect can separate WuXiBody from homodimer because TCR region cannot binds to KappaSelect.

mismatched byproducts [26]. As shown in Fig. 5B, the two heavy chain variable regions of a bispecific antibody are composed of VH2 and VH3 [26]. During the capture process, the VH2-VH2 homodimer does not no interact with the affinity resin and thus remains in the flow-through fraction [26]. In contrast, the VH3-VH3 homodimer binds tightly to the resin and is removed in stirp step (pH 2.5) [26].The bsAb has an intermediate binding strength, allowing it to be eluted under mild conditions (pH 4.0) [26].

Several bsAbs have a Fab*ScFv format, and because the ScFv-Fc construct lacks a complete CH1domain, ScFv-Fc homodimers can be

easily removed by employing affinity resins such as CaptureSelect CH1-LX. This is because ScFv - ScFv homodimers are unable to bind to the affinity resin and thus remain in the flow-through, whereas bsAbs can bind to the affinity resin (Fig. 5C) [16,27].

By exploiting the structural differences in light chains between bsAbs and homodimers, affinity resins targeting the light chains have also been proven effective in separating bsAbs from homodimers [28]. Specifically, KappaSelect affinity chromatography, which binds to the constant region of kappa LCs, facilitates the removal of homodimers incapable of incorporating kappa LCs [29]. A notable example is the WuxiBody platform, engineered by WuXi Biologics, that features a single kappa LC constant region, which distinguishes this method from homodimeric methods: the hole-hole homodimer, which CH1/CL region is replaced with T cell receptor (TCR), and the knob-knob homodimer, which contains two kappa LC constant regions [30]. Qin et al. have used KappaSelect affinity chromatography combined with linear pH gradient-based (pH 3.0–3.5) elution to effectively separate the hole-hole homodimers, and knob-knob homodimers from the WuxiBody (Fig. 5D) [31]. Similarly, Chen et al. have reported that Capto L affinity chromatography can remove the homodimers of WuxiBody through linear pH gradient (20 mM Na-citrate, pH 2.5–5.0) [32]. In addition, Dong et al. have demonstrated that the new generation of affinity chromatography resins MabSelect VL can efficiently remove the homodimers of the WuxiBody platform [33].

3.1.2. Ion exchange chromatography

Ion exchange chromatography with different modes (bind-eluate, flow-through, and weak binding mode) is an effective strategy for removing homodimers according to the isoelectric point (pI) difference between a bsAb and the homodimers [8]. The bind-eluate mode of ion exchange chromatography can be used to remove homodimers with higher or lower pI, compared with bsAbs [8]. A loading pH of 1–4 units away from the pI value of the bsAb is recommended for the use in ion exchange chromatography [1]. In cation exchange chromatography, by employing a loading pH lower than the pI value of bsAb, combined with a linear pH or salt gradient, the antibodies can be sequentially eluted in order from low to high pI values [1]. Several studies have shown that pH gradients exhibited superior separation efficiency for homodimers, particularly those with pI values that are close to the pI of the bsAbs, compared with salt gradients [34]. Sharkey et al. have reported that using the full pH gradient of a system (pH 4.0–11.0) could achieve baseline resolution of a bsAb (pI:8.94) from the homodimer (pI:7.99), but using a salt gradient (0–1 M NaCl) did not achieve resolution [35]. Further research by this group indicated that shallower pH gradient could provide a good separation of homodimer and bsAb [35]. This group used the cation exchange column MonoS 10/100 GL and a pH gradient (6.5–8.0) with a slop of 0.08 pH units/CV to completely remove two homodimers (with pI values of 8.95 and 8.36) [35].

In addition, the weak partitioning mode of ion exchange chromatography can be used to separate homodimers from the target antibody [36]. Kelley et al. have defined the weak partitioning mode that the values of products partition coefficient (Kp) range from 0.1 to 20 (the flow through mode needs to be used when $Kp \leq 0.1$) [36]. Using the weak partitioning mode, more protein binds to ion exchange resin than binds using the flow-through mode [5]. Our group has used the weak partitioning mode of anion exchange chromatography to remove the homodimer of a bsAb for the first time [5]. POROS 50HQ, an anion exchange chromatography resin from thermo Fisher Scientific, was used to purify a bsAb that adopt ART-Ig (pI slight lower than 7.0) [5]. Unexpectedly, the removal rate of the homodimer (pI slight lower than 6.0) can reach 99 % with a yield over 60 %, under the loading condition of pH 6.0–6.2, conductivity: 3.0–4.0 mS/cm [5]. To investigate the mechanism of homodimer removal, we test the Kp of bsAb under load condition is 0.6 and within the defined range for weak partitioning mode [5]. We assumed that when the pH of loading buffer is slightly lower than pI of bsAb, the bsAb bind to resin by the weak partitioning mode (the charge of its surface), and the homodimer is strongly bind to resin by binding mode [5]. At the onset of sample loading, the bsAb temporarily bind to the chromatography column, and with the number of antibodies increases, the homodimer binds more tightly than bsAb, giving the higher chance for bsAbs flowing through without binding [5].

3.1.3. Mix-mode chromatography

The purification of structurally complex bsAbs poses challenges because of the presence of homodimer impurities, which are difficult to adequately remove using conventional ion exchange chromatography [1]. Therefore, it is necessary to utilize chromatography modes with enhanced separation capabilities, such as mix-mode chromatography, for the purification process. Mix-mode chromatography allows the simultaneously provide multiple interaction mechanisms, including ionic interactions, hydrophobic interactions, hydrogen bonding, and sulfonate affinity, to effect separation [37]. Compared with ion exchange chromatography, mix-mode chromatography has several advantages, including a higher resolution and better salt tolerance [38]. Examples of mix-mode chromatography resins used for bsAbs purification, include Capto MMC, which couples cation exchange with hydrophobic interactions, and Capto Adhere, which combines anion exchange with hydrophobic interactions [38]. Currently, considerable research is focused on utilizing Capto MMC for the removal of homodimers from bsAbs [13]. Tang et al. have explored the ability of Capto MMC ImpRes to remove the hole-hole homodimer of a bsAb [13]. A linear pH gradient (pH 5.5–10.0) was used, and at pH 7.4, the eluate is enriched with the hole-hole homodimer [13]. Thus, employing a wash step at pH 7.4 prior to elution would be efficacious in eliminating the hole-hole homodimer [13]. Similarly, Chen et al. have developed an efficient purification process using Capto MMC, which reduced the content of the hole-hole homodimer from 10 % in the protein A eluate to <1 % in the Capto MMC eluate [39]. In addition, Fouque et al. have applied the mix-mode chromatography resin Toyopearl MX-Trp 650 M in the purification of a κλ−type bsAb [40]. The binding capacity of the κλ−type bsAb to the Toyopearl MX-Trp 650 M was found to lies between that of the homodimers κ−κ and λ−λ [40]. Consequently, a 75 mM NaCl elution was employed to specifically eluate the bsAbs, and the homodimers λ-λ and κ−κ were removed using the flow-through mode and a 500 mM NaCl wash step, respectively [40].

3.2. Removal of fragment

During antibody manufacturing processes, several factors, such as disulfide bond reduction and shear forces generated during the process, can lead to the formation of fragment-based impurities [1]. The presence of fragments such as half antibodies, light chain-missing fragments, and single-arm fragments, can impact the safety and efficacy of the bsAbs [1].This section examines the diverse chromatographic strategies employed to eliminate antibody fragment impurities.

3.2.1. Affinity chromatography

Fragment impurities, lacking a complete antibody structure, can be effectively removed through the use of appropriate affinity chromatography resins. Half-antibodies, which contain only one Fc domain, have weaker binding to protein A resin than the target antibody [10]. Hence, employing protein A chromatography combined with linear pH gradient elution is an effective method to separate half-antibodies from target antibodies [41]. Chen et al. have investigated the influence of incorporating different salts (NaCl, $CaCl_2$, and Arg-HCl) into the mobile phase on the resolution between half-antibodies and bsAbs [42]. Under these conditions (pH gradient: 5.5 to 2.8, salt concentration: 500 mM NaCl), all the half-antibodies present in the loaded sample (accounting for 13 % of the sample) were removed [42]. Additionally, Chen et al. have evaluated the ability of four different subdomain-specific affinity resin to remove half-antibodies [43]. Unlike protein A which can be used to remove half-antibody fragments effectively, the performance of four

subdomain-specific affinity resins (Capto L, CaptureSelect CH1-XL, CaptureSelect FcXP) was shown to be more dependent on structural differences between the bsAbs and the half-antibodies [43].

Employing LC-binding affinity resins is an effective strategy for the removal of light chain missing species, which lack one light chain compared with bsAbs [10]. For instance, Wang et al. have investigated the ability of Capto L to remove a light chain-missing fragment (contain only one kappa light chain variable region) of a Wuxi Body antibody (contain two kappa light chain variable regions) [44]. Utilizing Capto L combined with a pH linear gradient (5.0-3.2) allowed removal of 82.5 % of the light chain-missing fragment [44]. Similarly, Qin et al. have separated the kappa-LC missing fragment from the intact bsAb effectively using KappaSelect resin [31].

Single-arm antibody fragments are another type of fragment impurity, in which one Fab arm has been lost [45]. An affinity ligand that has affinity for the VH3 chain needs to be selected based on the structural characteristics of the VH3 chain [45]. A single-arm antibody fragment possessing only one VH3 domain has been shown to exhibit a reduced binding strength to MabSelect prismA relative to the bsAb [45]. This inherent difference enabled the use of MabSelect prismA chromatography to provide resolution between single-arm antibody fragments and the target antibodies [45]. After optimization, incorporating an additional washing step (pH 5.5) prior to the elution step (pH 4.2) was found to remove 46 % of the impurities [45]. In addition, further research demonstrated that, because of the utilization of cellulose fiber frameworks in Fibro prismA, the mass transfer performance was considerably enhanced, resulting in a greater ability to remove single-arm antibody fragments (the Fibro prismA eluate contained only 0.7 % of the single-arm antibody fragment [45].

3.2.2. Ion exchange chromatography and mix-mode chromatography

The prevailing strategy for bsAbs production involves structurally engineering antibodies at the design phase to manipulate their pI values, resulting in a shift in the pI of either the heavy or light chains within the bsAb constructs [1]. Consequently, charge-based purification is frequently employed to remove fragment impurities [1]. However, several studies have shown that ion exchange chromatography exhibited less consistent performance for removing fragment contaminants, compared with mixed-mode chromatography [8].

Compared with ion exchange, mixed-mode chromatography provides process stability over a broad range of operating conditions. Zhang et al. have conducted an evaluation of the removal of fragment impurities (LC-missing species) using anion exchange chromatography (POROS 50HQ) and the corresponding mixed-mode counterpart (Capto Adhere ImpRes) [46]. At low loading density (15 mg/ml), both anion exchange and mixed-mode resins were effective in eliminating the impurities [46]. However, at high loading density (60 mg/ml), anion exchange chromatography proved less efficient, whereas mixed-mode chromatography maintained effective removal of contaminants (85.9 %), with a yield of 64.0 % [46]. Hence, from the viewpoint of future large-scale industrial production, mixed-mode chromatography may have greater practical value.

Furthermore, in comparison with the weak binding mode of anion exchange, the binding-elution mode of mixed-mode chromatography has demonstrated superior efficacy in removing impurities with high pI values [1]. Wan et al. have investigated the efficiency of a cation mixed-mode chromatography resin (Capto MMC ImpRes) for removing fragment impurities (LC-missing species) [47]. Unlike anion exchange chromatography, fragment impurities with a high pI value exhibited a stronger binding affinity to Capto MMC ImpRes compared with the target bsAb [47]. As a result, during elution with a linear salt gradient, such impurities were eluted after the main peak [47]. Capto MMC demonstrated remarkable effectiveness in removing contaminants in this study; even at a loading capacity of 80 mg/ml, this resin achieved removal of 92.7 % of the contaminants [47]. In contrast, under a loading capacity of 60 mg/ml, the weak binding mode of anion exchange chromatography resulted in a removal of only 80 % of the impurities [47]. Thus, Capto MMC has good application potential for removing impurity fragments with high pI values. Additionally, to further enhance the resolution of mix-mode chromatography in the removal of fragment impurities, Wan et al. have optimized the elution mode for mix-mode chromatography [48]. The efficiency of the removal of fragment contaminants was compared between using a pH gradient (pH 6.0–8.5) versus a dual pH-salt gradient (pH 6.0–8.5; 0–250 mM NaCl) [48]. The pH gradient did not exhibit a significant effect on removing the single-arm byproduct. However, the dual gradient achieved a 66 % removal rate for the single-arm byproduct [48]. In addition, using the dual gradient removed half-antibodies and LC-missing species at rates of 98.2 % and 95.3 %, respectively [48]. Therefore, using a dual pH-salt gradient can improve the resolution of mix-mode chromatography in the purification process.

3.3. Removal of aggregation

Antibody aggregation has a highly detrimental effect on the manufacturing of bsAbs (Fig. 6). Aggregation can not only result in heightened immunogenicity, compromising the safety attributes of the product, but also leads to lower capacity and yield during protein A capture steps, thus affecting the robustness of the purification process [49]. Several studies have reported that aggregates originate from protein formation during the cell culture process as a result of both covalent and non-covalent interactions (2). Aggregates formed via non-covalent interactions are often reversible and can be converted back into monomers by adjusting the buffer composition, pH level, and ionic strength of the culture medium (2). For instance, Zhang et al. have demonstrated that incubating the cell harvest at pH 4.0 for 1 h, followed by changing the pH to 5.5, resulted in the conversion of approximately 50 % of the aggregates into the monomer form (2). In contrast, when dealing with aggregates formed through covalent bonds, various chromatographic methods must be employed to separate the impurities based on the physicochemical differences between the aggregates and the target antibody [8].

3.3.1. Affinity chromatography

Because both aggregates and bsAb possess similar structures, they can both be captured by affinity chromatography [1]. Research has shown that during the formation of aggregates, a corresponding steric hindrance is created, which results in the binding affinity of the aggregates to the affinity resin being weaker than that of the bsAb [50]. Consequently, a wash step containing additives prior to antibody elution can be employed to remove the aggregates [50]. Zhang et al. have reported that the addition of 5 % PEG and 500 mM calcium chloride into the wash buffer could reduce the content of aggregates from 20 % to 3%–4% during protein A affinity chromatography [50]. Moreover, because of the higher hydrophobicity of the aggregates compared with the antibodies, the inclusion of additives in the elution buffer can also enhance the resolution between the aggregates and antibodies [8]. Chen et al. have found that the amount of aggregates could be reduced from 66.5 % to 7.1 % after protein L chromatography with an addition of 100 mM Arg HCl in the elution buffer at pH 3.0 [32].

3.3.2. Mix-mode chromatography

The process of protein aggregation results in changes in the protein surface coverage, which subsequently gives rise to discernible differences in the surface charge of the aggregate compared with the monomers [49]. Consequently, ion exchange chromatography can be effectively utilized for the separation of aggregates from bsAb [51]. In addition, protein aggregation can increase the surface hydrophobicity, therefore hydrophobic interaction chromatography can be used to remove aggregates. Ion exchange chromatography provides relatively low resolution, however, hydrophobic interaction chromatography exhibits poor stability [8]. Therefore, the removal of aggregates can be a

Fig. 6. Schematic diagram of aggregate for bispecific antibodies.

highly challenging task.

Various studies have demonstrated the superior ability of mixed-mode chromatography in removing aggregates [38]. Chen et al. have investigated the effect of different elution modes on the removal of aggregates using mixed-mode chromatography [52]. Using Capto MMC with a linear gradient or stepwise elution could reduce the aggregate content from 20 % to 0.7 % and 2.6 %, respectively [52]. This result suggested that mixed-mode chromatography has the ability to remove aggregates regardless of the elution mode [52]. Additionally, Wan et al. have explored a dual pH-NaCl gradient elution mode for mix-mode chromatography. After purification using this method of mixed-mode chromatography, the aggregate content was reduced from 11.1 % to 1.2 % [48]. Zhang et al. have evaluated the ability of two mixed-mode chromatography resins to remove aggregates [53]. The results indicated that both Capto MMC and Diamond MMC Mustang exhibited excellent removal rates under stepwise gradient salt elution and the purity was improved from 69.6 % to 96.5 % and 97.4 %, respectively [53].

4. Process-related impurities removal strategies

The expression and purification of bsAbs can introduce a range of process-related impurities, including host cell proteins, viruses, and endotoxins. These process-related impurities can impact the safety of the antibody product. Typically, both upstream processes and downstream purification processes need to work in concert to control the content of process-related impurities. This section will focus on the strategies employed in downstream purification processes for the removal of process-related contaminants (Table 2).

4.1. Removal of host cell protein

Host cell proteins (HCPs), one of the types of process-related impurities, can potentially affect the safety and effectiveness of antibodies [54]. To reduce the risk associated with contaminants, the Food and Drug Administration (FDA) requires that the content of HCPs in the final product should be less than 100 ppm [55]. Various studies have indicated that controlling the content of HCPs requires a concerted effort involving both upstream and downstream processes to meet target specifications [56]. Optimizing upstream cell culture processes, such as adjustments to cell culture media compositions and bioreactor parameters, can effectively reduce the HCP levels in feedstocks, thereby, considerably increasing the efficiency of downstream purification [1]. Downstream processes employing chromatographic and filtration techniques are used to ensure the final product complies with regulatory limits regarding the HCP content [56]. This section focuses on the strategies employed in downstream purification for the efficient removal of HCPs.

4.1.1. Chromatography

In theory, protein A affinity chromatography should be highly efficient at removing HCPs because it has high specificity for the Fc region of antibodies [56]. However, extensive research has highlighted the issue of the co-elution of antibodies with HCPs, and even post-affinity chromatography, the HCP levels in the elution fraction can reach 1000 ppm [57]. Two key mechanisms primarily account for this co-elution phenomenon. First, chromatin plays a pivotal role in retaining HCPs by binding to both HCPs and antibodies, fostering heterogeneous aggregate formation. In addition, heterogeneous aggregates can non-specifically bind to protein A affinity resins through electrostatic and hydrophobic interactions [57]. Gagnon et al. have reported that implementing precipitation and membrane filtration steps ahead of protein A affinity chromatography effectively reduced the levels of heterogenous aggregates [58]. For instance, introducing caprylic acid pretreatment before protein A chromatography achieved a 2 log reduction value (LRV) removal [59]. Secondly, HCP-mAb association-mediated retention leads to the co-elution of HCP with the target antibody [56]. Several studies have demonstrated that elevating the pH value of the wash buffer (pH $>$ 4.5) reduced the HCP-mAb interactions, and including excipients, such as salts and solvents, in the wash buffer further decreased the content of bound HCPs [60]. Compared with the standard wash step, an enhanced wash step containing excipients can

Table 2
Summary of process-related impurities Removal Strategies for BsAbs.

Type of impurities	Removal Methods	Type of resin/filter	Removal Mechanisms	Reference
Host cell protein	Chromatography	Affinity chromatography	Affinity Differences	[57]
		Ion exchange chromatography	Differential surface charge	[60]
	Filter	Depth Filter	Size exclusion and adsorption	[63]
Virus	Low pH inactivation	/	/	[66]
	Chromatography	Protein A chromatography	Differential Affinity	[69,70]
		Anion exchange chromatography	The virus surface charge is opposite to that of the target antibody	[68,71]
		Hydrophobic interaction chromatography	Hydrophobicity Differences	[72]
		Mix-mode chromatography	Surface Charge and Hydrophobicity Differences	[73]
	Filter	Pegasus TM SV4	Size exclusion	[65]
		PlanovaTM 20 N	Size exclusion	[75]
Endotoxin	Chromatography	Anion exchange chromatography	Differential surface charge	[77]

achieve the high removal of HCPs (over 5 LRV) [60].

Polishing chromatography techniques, such as ion exchange chromatography combined with different modes, is also used to remove HCPs [61]. Because the pI values of most HCPs is lower than that of antibodies, the operating pH is set between the pI of the antibody and the HCPs [61]. In anion exchange chromatography, the HCPs are negatively charged and will bind to the resin, whereas mAbs are positively charged, and remain unbound in the flow-through fraction [62]. Conversely, in cation exchange chromatography, HCPs typically do not bind to the resin. The initial level of HCPs in the feed has a considerable impact on the removal of these impurities [62]. When the levels of HCPs in the feed is 10^2 ppm, cation exchange chromatography has a higher removal rate (2 LRV) for HCPs, compared with anion exchange chromatography (<1 LRV) [63]. However, when the HCP level ranges from 10^3 to 10^5 ppm, both chromatographic methods show comparable removal efficacy, with a performance of approximately 2–3 LRV [63].

4.1.2. Depth filters

Depth filters typically consist of a cellulose framework, auxiliary filter, and binders. The removal of HCPs with depth filters is by size exclusion and adsorption [64]. Following affinity chromatography, to maintain the stability of antibody molecules, it is common practice to adjust the pH to pH 5.50 [64]. Given that the isoelectric points (pI) of HCPs reside around 5.5, this adjustment process often results in the substantial precipitation of HCPs [64]. These precipitates can be removed through depth filtration [64]. Some HCPs with positive charges have exhibited high retention on negatively charged depth filter materials [64].

4.2. Removal of virus

For bispecific antibodies produced in mammalian cells, viral safety is a critical concern. According to the requirements of ICH Q5A, it is important to establish an effective virus inactivation and removal process in the purification of antibodies [65]. In the evaluation of virus clearance studies, two types of viruses: enveloped viruses (X-MuLv; Prv) and non-enveloped viruses (MVM) are employed as indicator viruses [65]. Lipid-enveloped viruses are sensitive to low pH conditions and can be inactivated through incubation under acidic conditions, whereas non-lipid-enveloped viruses are not sensitive to acidic environments and require removal via methods such as chromatography and filtration [65,66]. This section discusses viral removal strategies.

4.2.1. Low pH inactivation

Various studies have indicated that incubation at low pH (≤4.0) for 15–60 min is effective in inactivating enveloped viruses, such as X-MuLV [67]. For instance, Brorson et al. have reported that low pH inactivation at pH ≤ 3.8 and a temperature ≥14 °C for 30 min achieved ≥4.6 LRV clearance of X-MuLV [67]. In addition, to prevent the aggregation of antibodies caused by prolonged exposure to low pH conditions, it is essential to neutralize the solution after the incubation at low pH [67].

4.2.2. Chromatography

Various studies have shown that chromatography techniques, such as affinity chromatography, anion exchange chromatography, mix-mode chromatography, and hydrophobic interaction chromatography are effective strategies for the removal of viruses [65,68].

Protein A affinity chromatography, which is widely used in the capture and initial purification of bsAbs, has high affinity for bsAbs but does not necessarily capture viruses effectively [65]. In theory, Protein A affinity chromatography would suggest a potentially LRV for viruses. However, research has shown that protein A chromatography can exhibit relatively low virus clearance rates, with reported average LRVs of 2.98 for X-MuLV and 2.32 for MVM [69,70]. Bach and Connell-Crowley's study has suggested that the virus-mAb interaction, rather than chromatography parameters, may account for variations in viral clearance [71]. Consequently, it has been proposed that the LRV could be improved by adding interaction-disrupting additives, such as arginine or Triton, to the wash buffer [69].

Most model viruses, such as X-MuLV and MVM, have an pI value that is lower than that of bsAbs, therefore, anion exchange chromatography performed in flow-through mode can effectively remove both enveloped and non-enveloped viruses [65]. LRVs of 4.22 and 3.25 have been achieved for X-MuLV and MVM, respectively, using anion exchange chromatography [69]. Moreover, in several studies, AEX has exhibited robust viral clearance over a wide range of process parameters, even using the partitioning mode when the operating pH approached or exceeded the isoelectric point of the bsAb [65]. For instance, Iskra et al. have shown that using the partitioning mode, AEX had high LRVs for viruses, even under adverse conditions (e.g., samples containing a high percentage of aggregates) [72].

Hydrophobic interaction chromatography (HIC) is another viable option for viral clearance [65]. B Based on the hydrophobicity differences between the antibodies and viruses, HIC can be conducted using either flow-through or bind-elute modes [65]. The viral clearance ability of three different hydrophobic resins: POROS Ethyl; POROS Benzyl; and POROS Benzyl Ultra has been evaluated [73]. The results indicated that POROS Ethyl and POROS Benzyl could completely remove X-MuLV using the bind-elute mode, whereas POROS Benzyl Ultra was effective using the flow-through mode [73].

Mixed-mode resins, which combine the functional groups from ion exchange and hydrophobic resins, have demonstrated robust viral clearance across a broad range of pH and conductivity values [65]. The LRV of Capto Adhere (operated in flow-through mode at pH 6.75 and 10 mS/cm) for X-MuLV and MVM has been reported to have reached 4.5 and 5.8, respectively [74].

4.2.3. Virus filtration

Virus filtration can separate viruses (with an approximate size of 18–26 nm) from bsAb (with an approximate size of 12 nm) through a molecular exclusion mechanism, which has enabled the removal rate of viruses to reach 4 LRV while maintaining a high recovery rate [66]. Virus filters typically employ polymer materials, such as polyvinylidene fluoride (PVDF) and polyether sulfone (PES), which are characterized by low protein adsorption, resistance to organic solvents, and high flow rates [66]. For example, the Pegasus ™ SV4 filter (manufactured by Pall) uses PVDF as the membrane material, and has achieved a virus removal rate of 5.5 LRV for parvovirus under operating pressures ranging from 2.1 to 3.1 bar [66]. Moreover, Pegasus ™ Prime (using PES) can provide a removal rate of 4.0 LRV for parvovirus [66]. Additionally, the electrostatic nature of the membrane material used in virus clearance filters means that the ionic strength and pH value of the buffer solution can considerably influence the efficiency of the virus removal [66]. Strauss et al. have assessed the PlanovaTM 20 N filter by changing the pH and ionic strength of the feed solution [75]. According to this study, an oppositely charged virus (MVM) and a model filter (< pH 4) showed higher LRVs at lower ionic strengths [75]. However, when the virus and the model filter (< pH 8) had the same charge, a higher LRV was observed at higher ionic strengths [76].

4.3. Removal of endotoxin

Endotoxins, also known as lipopolysaccharides, are a component of the cell wall of Gram-negative bacteria [77]. The majority of endotoxins comprise an O-antigen region, a core oligosaccharide, and lipid A, with the toxicity of the endotoxin primarily associated with the hydrophobic lipid A moiety [77]. In the biopharmaceutical industry, it is crucial to control the endotoxin level in finished products to an extremely low threshold to prevent issues, such as the reduced biological activity, altered immunogenicity, and decreased stability of antibody drugs [77].

4.3.1. Chromatography

The pI values of most antibodies typically fall within the range of 7–9, whereas those of endotoxins are generally approximately 2 [78]. Under common buffer conditions, endotoxins carry a negative charge, which enables the removal of endotoxins from solution using ion exchange chromatography through either adsorption or permeation [78]. In ion exchange chromatography to remove endotoxins, materials, such as DEAE-based resins, DEAE membranes, and quaternary ammonium-based resins, can be employed, utilizing binding modes that facilitate the attachment of endotoxins to the resin, allowing only the target product to pass through [79]. In instances of high endotoxin loads (>1 μg/mL), reductions by up to five orders of magnitude can be achieved using ion exchange chromatography; however, under low endotoxin conditions (<10 ng/mL), it is typically necessary to maintain a low conductivity of the loading solution (e.g., <50 mM NaCl), which favors endotoxin adsorption [80]. Because of the opposite charges, endotoxin molecules can electrostatically bind to antibodies forming complexes that co-elute with the antibodies during their passage, elevating the endotoxin levels in the sample beyond acceptable limits [81]. Research has demonstrated that removing trace amounts of endotoxins from basic proteins is more challenging than from acidic proteins [78]. During ion exchange, the addition of agents to disrupt the interactions of these complexes can effectively reduce the endotoxin content in the final product [80]. Alkanediols have been proven effective in breaking the bonds in such complexes, and showed enhanced performance in cation exchange chromatography compared with anion exchange chromatography; however, the subsequent removal of the alkane diols poses an additional challenge [80].

5. Process integration

As shown in Fig. 7A, the classic purification process includes the capture of antibodies using affinity chromatography [1]. Low pH viral inactivation is used to inactivate viruses [67]. Anion exchange chromatography and cation exchange chromatography are typically employed to remove product-related impurities such as aggregates and homodimers [1]. Viral filtration is used to remove viruses, and diafiltration is utilized for buffer exchange [66]. However, as described in this review, with the emergence of new chromatography modes such as mix-mode chromatography, novel chromatography combination strategies have the potential to improve the purification of bispecific antibodies [1]. As shown in Fig. 7B, mix-mode chromatography can simultaneously replace both anion exchange and cation exchange chromatography, thereby reducing the number of chromatography steps [7]. Additionally, as illustrated in Fig. 7C, mix-mode chromatography has the potential to replace protein A, thus lowering costs [18].

6. Conclusion and outlook

BsAbs, which have the ability to simultaneously bind two antigen targets, have considerable therapeutic advantages in clinical applications, including enhanced efficacy and reduced side effects, compared with monoclonal antibodies. However, because of their intricate structure, the production process for bsAbs gives rise to various impurities. These impurities can be categorized into two types based on their origin: product-related impurities and process-related impurities. Surprisingly, there is currently a scarcity of comprehensive review articles systematically addressing the overall purification strategies for bsAbs. This

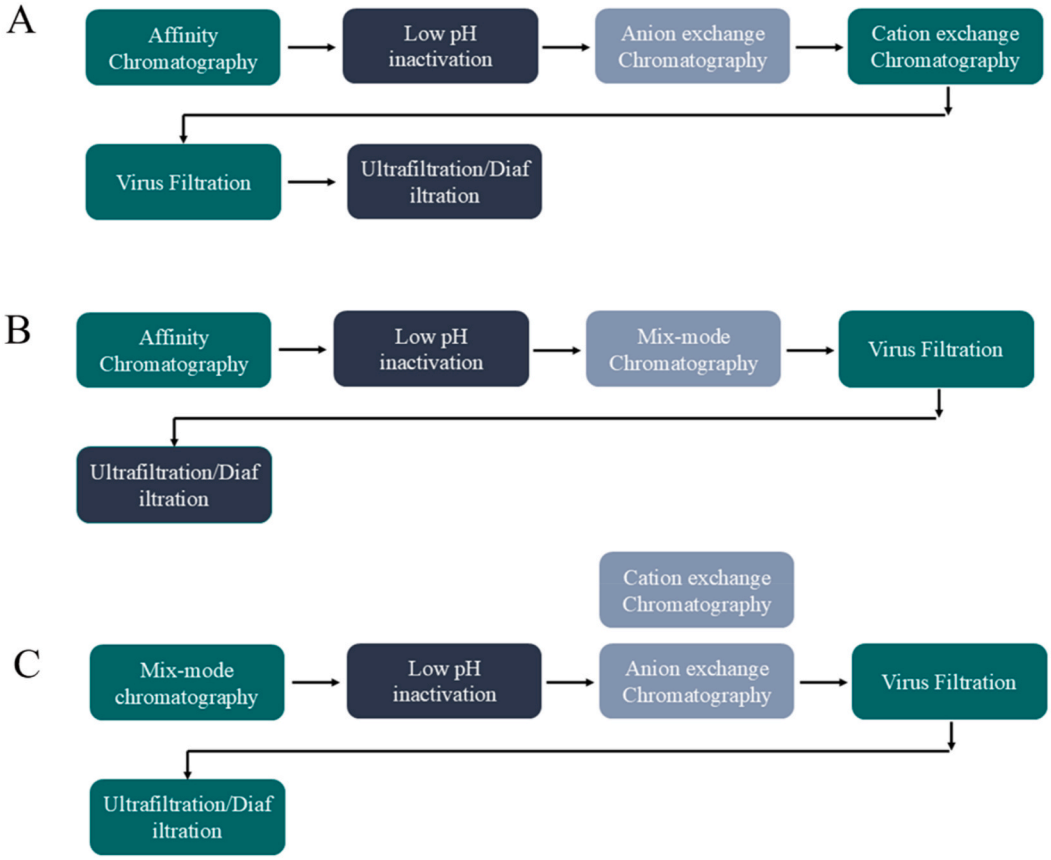

Fig. 7. Bispecific antibody purification Process Schemes. A. Classic purification process for bispecific antibodies B.Mix-mode Chromatography replacing ion exchange chromatography C. Mix-mode chromatography replacing affinity chromatography.

review aims to fill this gap by describing the capture of bsAbs, presenting a systematic overview of strategies that use different binding sites for capture, while also critically discussing the limitations of current Protein A chromatography, and envisioning prospective novel capture technologies for bsAbs. This review meticulously outlines the chromatographic strategies for the removal of the product-related impurities: homodimers; fragments; and aggregates. Turning to process-related impurities, the review delves into the application of chromatographic techniques, alongside filtration methodologies, tailored to eliminate HCPs, viral contaminants, and bacterial endotoxins. This review offers a holistic perspective for refining bispecific antibody purification processes, thereby contributing to advancements in ensuring the safety and efficacy of these biopharmaceuticals.

With the advancement of technology, particularly the integration of computer technology within the biopharmaceutical sector, the field of antibody purification is poised for breakthroughs in two key areas. First, the application of mechanism modeling through computer simulations for chromatography processes stands to revolutionize the field. This technology, leveraging deep learning among other methods, simulates the interactive forces at play during protein chromatography, thereby enhancing our understanding and guiding the development of more efficient downstream purification processes. Second, the advent of continuous flow processes based on digital twinning and real-time monitoring technologies promises a new era in manufacturing. These processes enable end-to-end continuous production of antibody therapeutics, reducing the losses associated with traditional batch processing and considerably boosting the production efficiency. By harnessing the power of digital replicas to mirror physical systems and employing in-line detection for immediate feedback and process control, this approach paves the way for optimized and streamlined manufacturing practices in the biopharmaceutical industry.

CRediT authorship contribution statement

Qian Li: Writing – original draft, Conceptualization. **Hongyang Zhao:** Writing – original draft. **Xiaoying Liang:** Investigation. **Qingquan He:** Visualization, Zicheng Wang, Visualization. **Guohong Qin:** Supervision. **GuoZhu Li:** Validation. **Dan Xu:** Conceptualization, Data curation.

Declaration of competing interest

The authors declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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